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Biological significance of allele specific loss of the p53 gene in breast carcinomas

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Abstract The p53 tumor suppressor gene has a central role in the defense against cancer, including breast cancer, and contains a polymorphic variant (Arg/Pro) at codon 72 that has been shown to have different biological properties regarding apoptosis and cell cycle arrest. Earlier studies have shown allele specific loss of heterozygosity (LOH) at this particular site and we aimed to investigate its biological relevance in codon 72 heterozygous breast cancer patients (i.e., survival and age of disease onset). 199 postmenopausal cases were analyzed for LOH using MegaBACE¹⁰⁰⁰ and statistics was performed using Statistical Package for Social Sciences. LOH was found in totally 124 (62.3%) patients and the Pro allele ($n = 103$) was significantly more often deleted compared to the Arg allele ($n = 21$) ($P = 0.001$). Patients with LOH of the Arg allele were diagnosed at an earlier age (mean age 62.5 years) than those with loss of the Pro allele (mean age 69.2 years) ($P = 0.011$). LOH of the Arg allele was also associated with worse survival ($P = 0.05$). LOH in comparison to ROH correlated significantly with increased S-phase fraction. Tumor size, stage or number of positive lymph nodes was not related to LOH. Our results and earlier findings suggest a selective loss of the Pro allele during carcinogenesis that might confer a growth advantage for cancer cells. On the other hand, it appears to be more harmful for patients to lose the Arg allele since we

found that loss of this allele was associated with earlier onset and worse prognosis.

Keywords Breast cancer · p53 · Codon 72 · Loss of heterozygosity · p53 Mutations

Introduction

The p53 tumor suppressor gene is located on chromosome 17p13 and has a central role in the defense against malignancy [1]. The p53 gene transcription is activated by genetic, environmental, and metabolic stimuli [2], which direct cells to different pathways including cell cycle arrest, DNA repair, and apoptosis. The protein is characterized by a common amino acid substitution resulting in a shift from an Arginine (Arg) to a Proline (Pro) at codon 72 [3]. These polymorphic variants have been shown to contain different biological properties as the Arg/Arg genotype has been reported to induce apoptosis more effectively than the Pro/Pro genotype [4–6]. This may be due to enhanced mitochondrial localization of the p53 protein in cells harboring the Arg/Arg genotype or that the inhibitor of p53, iASPP, bind to and regulate the activity of p53Pro72 more efficiently than that of p53Arg72 [7]. The Pro/Pro genotype, on the other hand, appears to induce higher level of G1 arrest than does the Arg/Arg genotype [6]. The contribution of codon 72 to breast cancer risk has been investigated by several authors but results are diverse so its implication remains uncertain [8–13].

The significance of p53 in carcinogenesis is manifested by the frequent genetic alterations in human cancers mostly found as mutations within the DNA binding domain but also as loss of heterozygosity (LOH) in the loci of the gene. Several studies have reported LOH at codon 72 with a

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preferential loss of the Pro allele both in breast cancer as well as in other malignancies [14–16]. It has also been described that the p53 genotype in addition to LOH status of codon 72 influences the prognosis in cancer. In breast cancer, Tammiska et al. [8] and Toyama et al. [17] have studied this polymorphism in relation to prognosis and they found a reduced survival for homozygous carriers of the Pro allele. However, the influence of allele specific loss in Arg/Pro heterozygotes on survival in breast cancer patients seems uncertain since only a few reports with limited populations has been published [14, 18].

The aim of the present study was to investigate allele specific LOH at codon 72 of the p53 gene in breast cancer patients heterozygous for the codon 72 polymorphism. We further investigated p53 mutations in cases showing loss of either Pro or Arg allele. p53 data were evaluated in relation to prognosis, age of disease onset and the association to histopathological features of the breast tumor.

Patients and methods

From the primary cohort of 677 postmenopausal patients with a stage II and III disease 495 cases were genotyped in codon 72 (Pro/Arg) [19]. Results revealed 199 heterozygous patients that were selected for the study of allele specific LOH. Information of tumor size, lymph-node status, S-phase fraction and tumor stage is shown in Table 1. DNA was isolated from frozen tumor tissues. All patients were estrogen-receptor (ER) positive and had received adjuvant tamoxifen treatment. The patients with a mean age of 69 years (range 50–96 years) were diagnosed in the South East Health Care region of Sweden between 1986 and 1997. The mean follow up time was 7.3 years (range: 0.04–17.9 years). As reference for loss/retention of heterozygosity at the Arg or Pro allele DNA from 30 heterozygous blood donors included in a population based DNA bank were used.

Loss of heterozygosity (LOH) assay

Genomic DNA from frozen tumor tissues and blood samples were isolated using Wizard[®] SV Genomic DNA

Table 1 Information of tumor size (mm), stage, nodal involvement and s-phase fraction according to allele specific loss at codon 72

	Tumor size		Stage		Nodal status		S-phase	
	≤25	>25	II	III	0	≥1	≤7	>7
ROH	51	24	69	6	25	50	46	22
LOH Arg	11	9	19	2	3	18	6	13
LOH Pro	55	48	95	8	39	64	45	49
<i>P</i>	0.14		0.83		0.11		0.006	

P-values were calculated by Chi-square test

Purification System (Promega Corporation, Madison, WI, USA). The codon 72 polymorphic site of the p53 gene was amplified using a pair of forward and reverse primers with the reverse primer labeled with FAM dye (5'-GAAGACCCAGGTCCAGATGA-3', FAM 5'-CTGCCCTCCTAGGTTTTCTG-3', respectively). The PCR reaction was carried out in a volume of 20 µl containing 25–50 ng of DNA, 11.1 µl H₂O, 2 mM MgCl₂, 0.2 mM dNTP, 1 µM of both forward and reverse-primer, and 0.1 µl (0.025 U/l) Tag polymerase (Bio labs Inc.). Amplification was performed under following conditions: initial denaturizing at 94°C for 2.5 min, followed by 38 rounds of thermal cycling (94°C for 30 s, 57°C for 30 s, 72°C for 1 min, and a final extension at 72°C for 5 min). The PCR product (152 bp) was cleaved at the polymorphic position by the enzyme BstU I (Biolabs Inc.) at 60°C for 5 h. The cleaved product was then analyzed in the MegaBACE¹⁰⁰⁰. The intensity of peaks in the MegaBACE1000 histogram represented the amount of each allele.

Mutation analysis

Tumors expressing LOH at codon 72 were analyzed for mutations in exon 5–8 of the p53 gene. PCR products of exons were labeled by inclusion of [³³P] dATP through 20 cycles of PCR. Electrophoresis was performed on a non-denaturing 6% polyacrylamide gel at 6 W for 14–16 h. The dried gel was exposed on an X ray film. Samples showing mobility shifts were collected from the gel and amplified by 15 cycles of PCR. DNA sequencing was performed using Big Dye version 1.1 and the ABI PRISM[®] 3100 Genetic Analyzer (Applied Biosystems).

Statistical analyses

Statistical Package for Social Sciences (SPSS) Advanced ModelsTM 16.0 software was used for the statistical analyses. The survival curve of recurrences was estimated using the Kaplan-Meier method and the differences in survival rates for patients was assessed by Log-Rank test. An univariate Cox proportional hazard model was used for the estimation of the hazard ratio (HR) comparing cases with different LOH status. One-way analysis of variance (ANOVA) was utilized for comparing mean age at diagnosis. We used Chi-square test to compare the distribution of S-phase fraction, number of positive lymph nodes, stage and tumor size according to the LOH data, as well as the difference in the distribution of LOH.

To evaluate whether cases had retention of heterozygosity (ROH) or LOH the ratio (Pro/Arg) of intensity peaks from MegaBACE¹⁰⁰⁰ histograms was used (Fig. 1). First, to obtain an estimate of the normal variation for the different ratios we calculated the standard deviation in the

control population. Next, the limits between ROH and LOH were defined as the mean ratio of the controls plus/minus standard deviation (SD). Cases with a Pro/Arg ratio below the SD were judged as having loss of the Pro allele. Those with a ratio above the SD were considered to have a loss of the Arg allele. Finally, the values within SD were defined as ROH.

Results

In the genotyped population 209 cases were homozygous for the Arg allele, 48 homozygous for the Pro allele and 238 were heterozygous. Homozygous cases of the Arg allele were diagnosed significantly earlier (mean = 65.4) than heterozygous patients (mean = 67.5) ($P = 0.026$). No such difference in age at diagnosis could be seen in Pro/Pro cases. No statistical difference in prognosis was found between genotypes. Tumor size and the number of positive lymph nodes were equally distributed among the different genotypes.

In the control population mean ratio of Pro/Arg was 1.5, median 1.55, range 1.1–2.2 and the standard deviation 0.3. For breast cancer cases a ratio less than 1.2 was considered as loss of the Pro allele whereas a ratio above 1.8 defined loss of the Arg allele. Ratios between 1.2–1.8 represent patients with ROH (Fig. 1). 124 out of 199 codon 72 heterozygous patients (62%) demonstrated LOH and the Pro allele ($n = 103$) was shown to be considerably more often lost compared to the Arg allele ($n = 21$) ($P = 0.001$). The p53 gene mutations were found in 15 cases of which 12 expressed loss of the pro allele (Table 2). Patients with LOH at the Arg allele were diagnosed at a significantly earlier age (mean 62.5 years) than those with loss of the Pro allele (mean 69.2 years) ($P = 0.011$). In addition, loss of Arg was also associated with worse survival ($P = 0.05$) (Fig. 2). Calculated Hazard Ratio for LOH of the Arg allele was 2.2 (CI 1.03–4.69). LOH in comparison to ROH was

Table 2 Mutations in the p53 gene among patients showing LOH of either Pro or Arg allele in codon 72

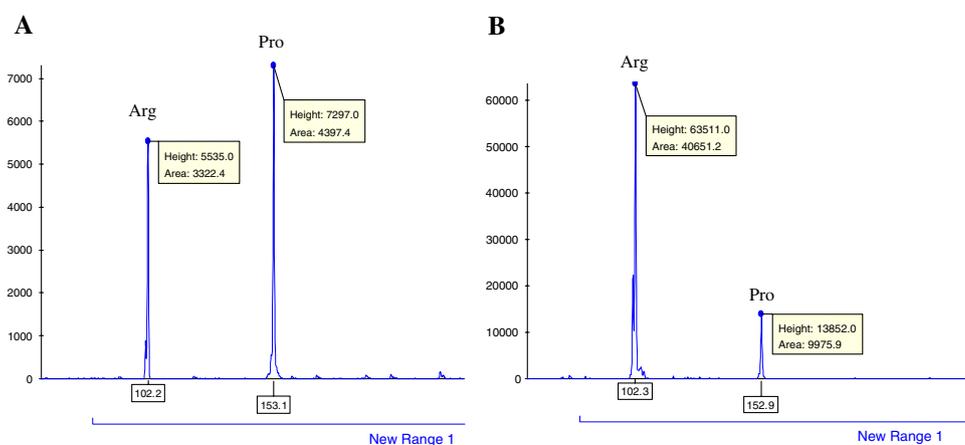
Case no.	Allelic loss Codon 72	Mutation	
		Codon	Description
241	Pro	278	Pro-Ala
253	Pro	288	Insertion Stop
258	Pro	243	Met-Leu
360	Pro	181	Arg-Pro
365	Arg	243	Met-Leu
366	Arg	135	Cys-Trp
416	Pro	194	Leu-His
450	Arg		Complex deletion in exon 6
464	Pro	179	His-Leu
491	Pro	195	Frame shift
550	Pro	215	Ser-Stop
556	Pro	175	Arg-His
605	Pro		Complex deletion in exon 6
621	Pro		Complex deletion in exon 6
641	Pro	285	Glu-Lys

associated with increased S-phase fraction ($P = 0.006$) (Table 1). When loss at each allele was compared to ROH, an increased S-phase fraction was associated with both the Pro ($P = 0.02$) and Arg ($P = 0.01$) allele. No association between LOH at codon 72, tumor size, stage or lymph node status was found (Table 1).

Discussion

In the present investigation we found that LOH occurred significantly more often at the Pro allele compared to the Arg allele in codon 72 heterozygous breast cancer patients. This phenomenon has also been seen in colorectal cancer [15], squamous cell carcinomas [20], ovarian carcinomas

Fig. 1 Histograms of the Pro and Arg allele in a control (a) and a breast cancer case (b). **a** Illustrates retention of heterozygosity (i.e., the calculated ratio of Pro/Arg is within the limits that define ROH). **b** Illustrates a tumor where the proportion of the Pro allele is small and thereby the ratio of intensity peaks judges the case as loss of the Pro allele



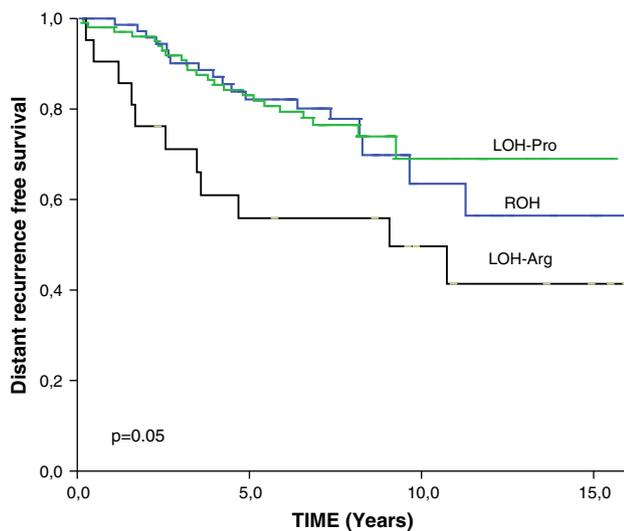


Fig. 2 Distant recurrence-free survival in Pro/Arg heterozygous breast cancer patients in relation to LOH status in codon 72

[21], and lung cancer [22], as well as in cancers involving esophagus, and the urinary tract [23, 24]. The biological significance of these univocal findings seems unclear. However, several reports [24–26] have indicated that the Arg allele is a target for mutation in cancers arising from Arg/Pro heterozygous patients and that the mutated *p53* Arg but not the Pro allele is capable of inactivating *p73* dependent apoptosis [27]. A possible mechanism for this inactivation might be that the mutant Arg allele is able to form complexes with the *p73* protein thus influencing its DNA binding capacity and transcriptional activity. In the present study most mutations were found in cases with loss of the Pro allele, however, this overrepresentation might be a consequence of the prevalent loss of the Pro allele and the fact that only cases with LOH were subjected to mutation analysis. In non-small lung cancer Szymanowska et al. [28] investigated the genotype of codon 72 and mutations in the *p53* gene. They found a tendency towards more frequent mutations in Pro carriers compared to Arg72 homozygous. A significant result in the same direction was found by Hu et al. [29] in non-small cell lung cancer showing that *p53* mutations were associated with the number of codon 72 Pro alleles.

The knowledge of allele specific LOH in Arg/Pro heterozygous breast cancer patients in relation to prognosis is relatively scarce although a few reports with limited number of cases have been published [14, 18]. A report by Kyndi et al. [18] including 68 heterozygous breast cancer patients is in concordance with the present study, demonstrating a worse prognosis in patients with loss of the Arg allele. The opposite was found by Bonafe et al. [14] in a cohort of 29 heterozygous breast cancer patients. In other studies investigating the codon 72 Arg/Pro genotype and

prognosis in breast cancer Tommiska et al. [8], Toyama et al. [17] and Xu et al. [30] report a significantly reduced survival for patients homozygous for the Pro allele. This is to some extent in agreement with our findings since loss of the Arg allele in heterozygous cases as well as homozygosity for the Pro allele both point towards an important role of the Arg allele in order to obtain an improved prognosis. In addition, its implication in breast carcinogenesis is also strengthening by the association between earlier onset of breast cancer and LOH of the Arg allele in the present cohort. A similar tendency of younger age at diagnosis in carriers of the Pro allele was also found by Tommiska et al. [8] in hereditary breast cancer.

The role of codon 72 variants in apoptosis as well as its interplay with treatment may have a significant influence on our prognostic findings. Functional data from in vitro studies suggests that these two protein variants act differently and the Arg/Arg genotype has been reported to induce apoptosis more efficiently than the Pro/Pro genotype [5–7]. However, until recently the majority of in vitro studies have been performed under normoxic conditions even if the most likely environment surrounding cancer cells in vivo is hypoxia. Vannini et al. [31] therefore investigated the influence of hypoxia in relation to the *p53* codon 72 polymorphism and observed that cells transfected with the Pro allele had higher level of cell death compared to cells with the Arg allele. They also found that Arg transfected cells expressed higher levels of the Breast Cancer Resistance Protein-I (BCRP-I), which is a hypoxia response gene known to be involved in resistance to cytotoxicity induced cell death.

The influence of *p53* genotype and mutational status in response to chemotherapy is complex which has been shown by Xu et al. [32] in breast cancer and by Bergamaschi et al. [27] in advanced head and neck tumor. In breast cancers homozygous for the Pro allele patients showed less response to anthracycline-based neoadjuvant treatment whereas in advanced head and neck tumors patients with a mutant Arg allele who were treated with cisplatin-based chemo-radiotherapy had lower response rate than those with a mutation at the Pro allele. Vannini and colleagues [31] also investigated the relation of *p53* codon 72 polymorphism and clinical response to chemotherapy in 40 breast cancer cases and found that the Arg homozygous status was an independent indicator of worse prognosis. In breast cancer patients treated with 5-Fluorouracil and Mitomycin Nordgaard et al. [33] found no association between treatment response and genotype of codon 72 however; they did find that 5-Fluorouracil metabolizing enzymes were regulated in an allele specific manner. This dosage response was strengthening by the in silico-binding site for *p53* found in promoter region of the majority of the metabolic genes that were regulated.

We found a non-random loss of the codon 72 Pro allele, implying the existence of a selective pressure during carcinogenesis that confers a growth advantage to cells lacking the Pro allele. We also found that earlier age of onset was associated with loss of the Arg allele. These results were achieved from primary tumors without any influence of adjuvant treatment, possibly showing a picture of the natural history of carcinogenesis. The worse prognosis associated with loss of the Arg allele is somewhat more complex to interpret since recurrence free survival could be influenced by the intratumoral environment, genotype, mutational status and adjuvant treatment.

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